Remarks

The claims have been amended to overcome the objections.

Applicants traverse the rejections of the present claims for obviousness and contend that the Examiner has engaged in an impressible hindsight reconstruction of the prior art. Moreover, the Examiner has not demonstrated the requisite motivation or reasonable expectation of success for the present invention. Finally applicants attach hereto commentary and data by the inventors that evidence non-obviousness. Specifically, the remarks evidence that the combination of references cited by the Examiner do not demonstrate a reasonable expectation of success. If the Examiner requires, applicants can submit the remarks in the form of 132 Declaration.

I. Response from Inventors to Obviousness Rejection

It should be understood that the production of antibodies against any foreign molecule, even table salt is an inherent property of the animal. But obtaining antibodies to develop test systems especially with small molecules involves derivatisation of the antibody. In the present study the antibodies derived against the said pesticides molecule involved synthesis and identification of right conjugation to obtain a correct epitope specific antibodies, which are able to recognize the pesticide of interest. The process of developing said antibodies involved the following requisites which have not been performed in any of the prior art and neither the present process has been suggested in the prior art:

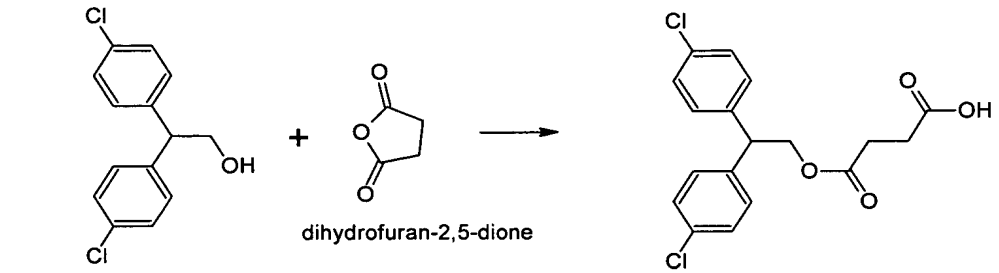
(A). Synthesis of haptens and their active esters:

(a) Synthesis of DDT-OH hapten 4-[2,2-bis(4-chlorophenyl)ethoxy]-4-oxobutanoic acid (DDT-1):

DDT-OH [2,2-Bis (4-chlorophenyl) -1,1,1- trichloroethanol] was reacted with an excess of succinic anhydride in pyridine to obtain the above hapten after work-up. This was reacted with N-hydroxy succinimide in presence of *N,N'*-dicyclohexylcarbodiimide (DCC) in

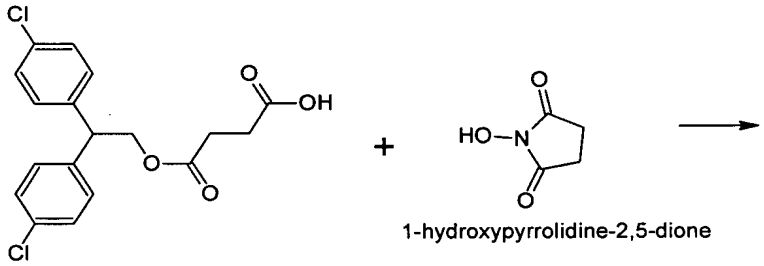
dichloromethane to obtain the active ester of the hapten viz., 2,2-bis(4-chlorophenyl)ethyl 4-[(2,5-dioxopyrrolidin-1-yl)oxy]-4-oxobutanoate that was used to conjugate the hapten to carrier proteins and horse-radish peroxidase enzyme. (Scheme-1)

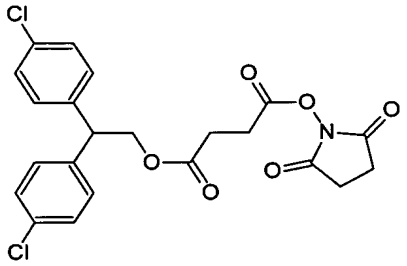
Scheme-1: Synthesis of DDT-OH hapten (DDT-1) and its active ester:



2,2-bis(4-chlorophenyl) ethanol

4-[2,2-bis(4-chlorophenyl)ethoxy]-4-oxobutanoic acid





2,2-bis(4-chlorophenyl)ethyl 4-[(2,5-dioxopyrrolidin-1-yl)oxy]-4-oxobutanoate

(b) Synthesis of DDA-GABA hapten 4-{[bis(4-chlorophenyl)acetyl]amino}butanoic acid (DDT-2):

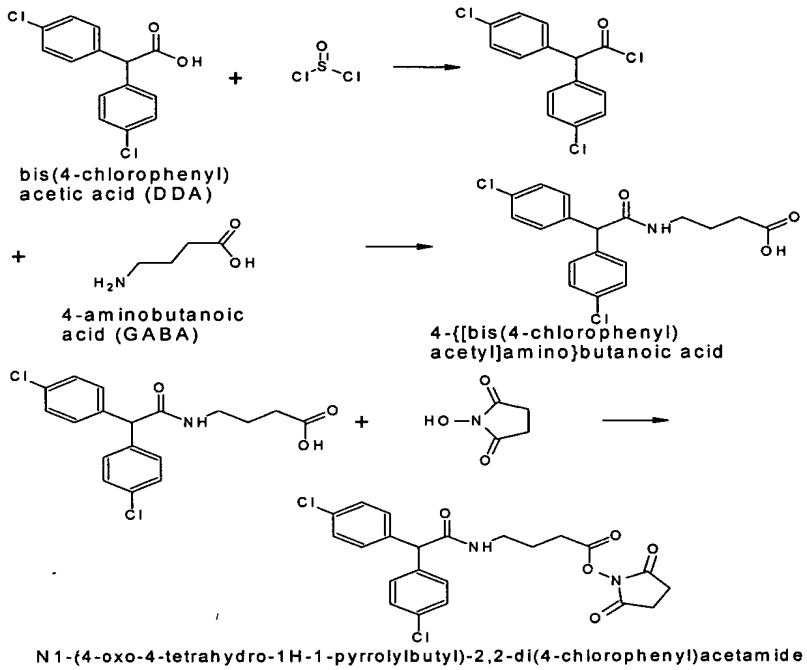
DDA was converted to its acid chloride by reaction with thionyl chloride. The acid chloride was dissolved in benzene and reacted with γ-aminobutyric acid (GABA) dissolved in

1 M NaOH. TLC analysis of the crude product showed a single spot at R_f 0.17 along with traces of unreacted DDA. About 400 mg hapten were obtained this way.

Crude DDA-GABA was subjected to column chromatography using ethyl acetate + petroleum ether + acetic acid (50 + 49.9 + 0.1) eluent. Thirteen fractions of about 10 mL each were collected and analyzed by TLC. Fractions 7-13 contained pure DDA-GABA hapten. They were pooled and solvent evaporated off to get pure DDA-GABA hapten.

DDA-GABA was reacted with N-hydroxysuccinimide in dichloromethane in presence of dicyclohexylcarbodiimide and dimethylaminopyridine as catalyst. TLC analysis showed spot at R_f 0.2 using ethyl acetate + petroleum ether + acetic acid (50 + 49.9 + 0.1) eluent. The reaction mixture was filtered to remove dicyclohexylurea and dichloromethane evaporated off to get pure active ester of DDA-GABA viz., N1-(4-oxo-4-tetrahydro-1H-1-pyrrolylbutyl)-2,2-di(4-chlorophenyl)acetamide. This was used to conjugate the hapten to carrier proteins and HRP (Scheme-2).

Scheme-2: Synthesis of DDA-GABA hapten (DDT-2) and its active ester



(c) Synthesis of octachloro cyclic hapten: 4-[(1,3,5,7,8,9,10,10-octachlorotricyclo[5.2.1.$0^{2,6}$]dec-8-en-4-yl)oxy]-4-oxobutanoic acid for endosulfan (Endo-1):

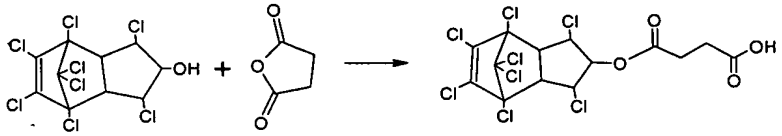
Heptachlor was dissolved in glacial acetic acid. tert-Butyl hypochlorite was dissolved in glacial acetic acid and added to the first solution. The mixture was refluxed on a water-bath for 1 hour. Fine crystals of acetyl-chloro derivative of heptachlor separated out. They were filtered, washed with acetone and air-dried. This was suspended in methanol, dry HCl gas was passed and the mixture was refluxed for 30 min. HCl gas was passed again and the mixture was refluxed overnight. Methanol was evaporated off and the mixture was dissolved in diethyl ether. The organic layer was washed with water, bicarbonate, dried over calcium chloride and ether evaporated off to get the pre-hapten 1,3,4,5,6,7,8,8-Octachloro-2-hydroxy-4,7-methano-3a,4,7,7a-tetrahydroindane. The above hydroxy compound was succinylated in pyridine in presence of dimethylaminopyridine overnight and the product worked up in ethyl

acetate to get the hapten. The product was very pure as checked by TLC in ethyl acetate + petroleum ether + acetic acid (50 + 49.9 + 0.1) eluent.

1. (d) Preparation of Active Ester of the Octachloro cyclic hapten for endosulfan

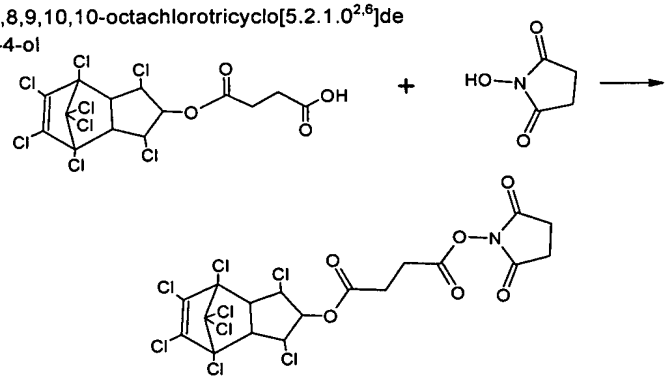
The hapten was dissolved in dichloromethane and N-hydroxysuccinimide was added. Dicyclohexylcarbodiimide was added followed by dimethylaminopyridine. Dicyclohexylurea was filtered off and dichloromethane was evaporated to get the active ester of endosulfan hapten viz., [1,4,5,6,7,7-hexachloro-3-(hydroxymethyl)bicyclo[2.2.1] hept-5-en-2-yl]methyl 4-[(2,5-dioxopyrrolidin-1-yl)oxy]-4-oxobutanoate. (Scheme-3).

Scheme-3: Synthesis of the octachloro cyclic hapten for endosulfan:



4-[(1,3,5,7,8,9,10,10-octachlorotricyclo[5.2.1.02,6] dec-8-en-4-yl)oxy]-4-oxobutanoic acid

1,3,5,7,8,9,10,10-octachlorotricyclo[5.2.1.$0^{2,6}$]dec-8-en-4-ol



[1,4,5,6,7,7-hexachloro-3-(hydroxymethyl)bicyclo[2.2.1] hept-5-en-2-yl]methyl 4-[(2,5-dioxopyrrolidin-1-yl)oxy]-4-oxobutanoate

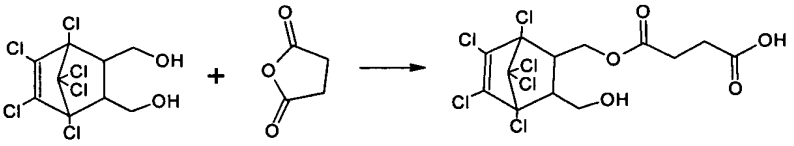
(e) Preparation of Endo-diol Hapten: 4-{[1,4,5,6,7,7-hexachloro-3-(hydroxymethyl) bicyclo2.2.1]hept-5-en-2-yl]methoxy}-4-oxobutanoic acid (Endo-2)

Endodulfan diol {[1,4,5,6,7,7-hexachloro-3-(hydroxymethyl)bicyclo[2.2.1]hept-5-en-2-yl]methanol} was reacted with succinic anhydride in pyridine in presence of

dimethylpyridine overnight. TLC analysis showed formation of the succinylated hapten along two byproduct impurities along with the unreacted endosulfan diol. The mixture after workup was subjected to column chromatography using ethyl acetate + petroleum ether + acetic acid (50 + 49.9 + 0.1) eluent. 21 Fractions were collected and analyzed by TLC using the same solvent system. Fractions 7-13 contained the pure hapten.

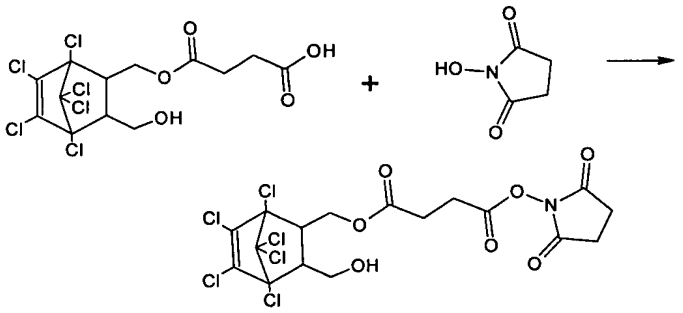
The hapten was dissolved in dichloromethane and reacted with N-hydroxysuccinimide in presence of dicyclohexylcarbodiimide and dimethylaminopyridine catalyst to get the active ester of endodiol hapten viz., [1,4,5,6,7,7-hexachloro-3-(hydroxymethyl) bicyclo[2.2.1]hept-5-en-2-yl]methyl 4-[(2,5-dioxopyrrolidin-1-yl)oxy]-4-oxobutanoate (Scheme-4)

Scheme-4: Synthesis of the Endo-diol hapten for endosulfan (Endosulfan-2):



[1,4,5,6,7,7-hexachloro-3-(hydroxy methyl)bicyclo[2.2.1]hept-5-en-2-yl] methanol

4-{[1,4,5,6,7,7-hexachloro-3-(hydroxymethyl)bicyclo 2.2.1]hept-5-en-2-yl]methoxy}-4-oxobutanoic acid



[1,4,5,6,7,7-hexachloro-3-(hydroxymethyl)bicyclo[2.2.1] hept-5-en-2-yl]methyl 4-[(2,5-dioxopyrrolidin-1-yl)oxy]-4-oxobutanoate

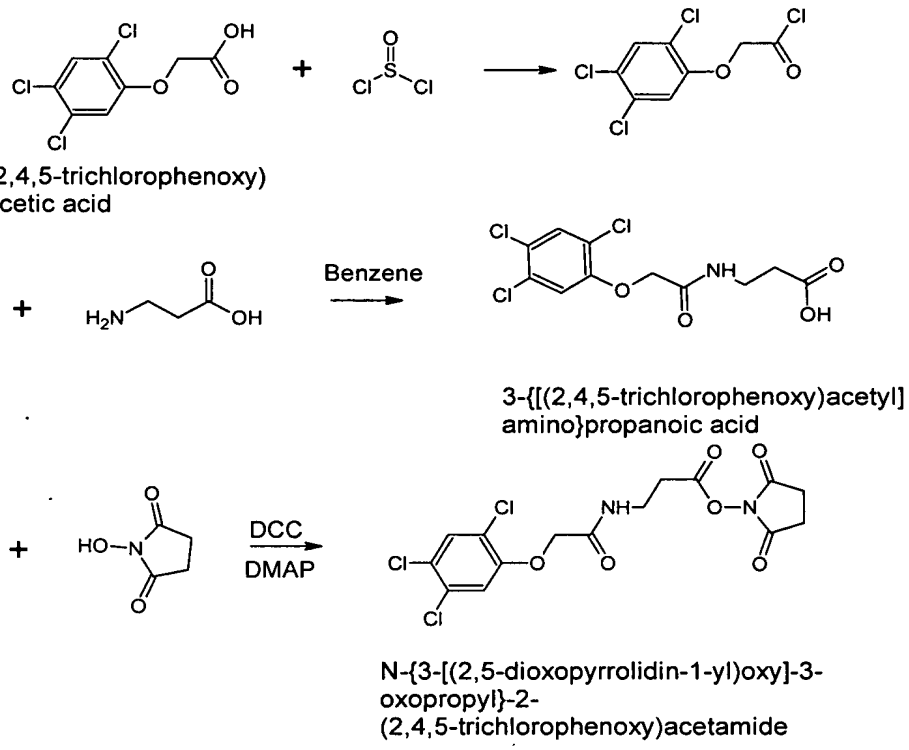
(f) Synthesis of HCH haptens: (a) 3-{[(2,4,5-trichlorophenoxy)acetyl]amino}propanoic acid (HCH-1) and (b) 4-{[(2,4,5-trichlorophenoxy)acetyl]amino} butanoic acid (HCH-2):

2,4,5-Trichlorophenoxyacetic acid was refluxed with thionyl chloride (72 mL) and the excess thionyl chloride distilled off to get the acid chloride. Acid chloride was dissolved in benzene and β-alanine/GABA-Na was added at 0 °C and the mixture was stirred overnight. Benzene was distilled off and the mixture was acidified to obtain a solid that was filtered and washed with water until the filtrate was neutral. HCH-1/HCH-2 was obtained (Scheme-4 and 5).

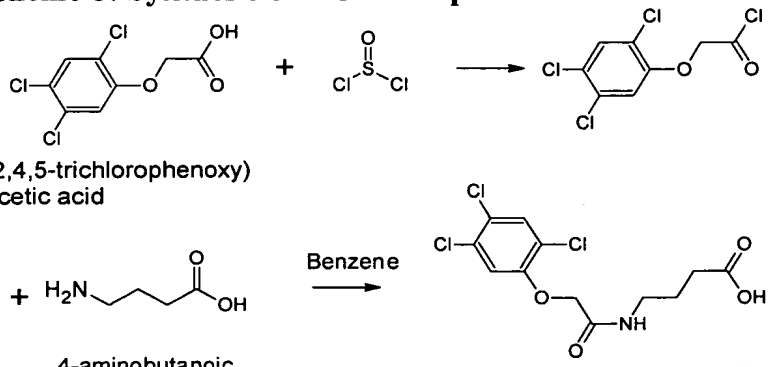
The hapten 4-{[(2,4,5-trichlorophenoxy) acetyl]amino} butanoic acid was characterized and converted to its active ester *N*-{4-[(2,5-dioxopyrrolidin-1-yl)oxy]-4-oxobutyl}-2-(2,4,5-trichlorophenoxy)acetamide in dimethylformamide as given below: 4-{[(2,4,5-trichlorophenoxy)acetyl]amino}butanoic acid and N-hydroxysuccinimide were dissolved in dimethylformamide. *N,N'*-dicyclohexylcarbodiimide was added to the solution and incubated at RT overnight. Formation of the active ester viz., N-{3-[(2,5-dioxopyrrolidin-1-

yl)oxy]-3-oxopropyl}-2-(2,4,5-trichlorophenoxy)acetamide was tested by TLC using MeOH + $CHCl_3$: 10 + 90 eluent. The R_f for the hapten was 0.32 and that of the active ester was 0.80. No starting material impurity was observed.

a. Scheme-4: Synthesis of HCH-1 hapten and its active ester



Scheme-5: Synthesis of HCH-2 hapten and its active ester:



N-{4-[(2,5-dioxopyrrolidin-1-yl)oxy]-4-oxobutyl}-2-(2,4,5-trichlorophenoxy)acetamide

II. Preparation of conjugates of haptens with proteins and HRP:

Carrier Proteins ovalbumin (OVA) and bovine serum albumin (BSA) were dissolved in phosphate buffer and 20- and 40-fold molar excess active esters of haptens were added to the protein solutions at low temperature. The hapten-protein conjugates were dialyzed in phosphate buffered saline against three changes.

Horse-radish peroxidase (HRP)-hapten conjugates were prepared in a similar way. The carrier protein conjugates were stored below 0 °C and the HRP conjugates were stored at ca. 8 °C.

Further the inventors wish to respectfully submit that out of the four conjugates viz., DDT-OH conjugated to OVA and BSA, DDA-GABA conjugates to OVA and BSA, DDT-OH-OVA conjugate gave the best antibodies in rabbits, while in the chicken DDT-OH-BSA gave good antibodies. This is indicative of the varied reaction to the same antigen depending on the animal system. This also proves that rabbit antibodies against HCH produced by Beasley are not similar to the HCH antibodies of chicken. Also, the hapten protein conjugate used by Beasley et al is not the BSA conjugate as used in this study.

The Trichlorobenzene hapten for the immunoassay of HCH synthesized by Beasley *et al.* was conjugated to OVA and keyhole limpet haemocyanin (KLH) and not to BSA for raising antibodies in rabbits. When trichlorobenzene containing a γ-aminobuyric acid spacer arm that means increase of a methylene group was synthesized by one of the inventors (Scheme-5), and rabbits were immunized with conjugates of this hapten (HCH-2) we found that the antibodies recognized the hapten itself but not trichlorobenzene (the target molecule) in one of our trials. (A. Pasha and Amita rani (2003), Unpublished work). Both HCH-1-HRP and HCH-2-HRP were employed as tracers but it did not help.

On the other hand in the present study inventors have conjugated HCH-1 hapten to BSA and found that this was most useful for raising antibodies in chicken. The sensitivity of the assay was higher than the published method in which rabbits were used to raise antibodies. This hapten contains a β-alanine spacer arm. This is difference is because of the basic antigen. The inventors did not arrive at this inference only much of experimentation.

Endo-1I hapten for endosulfan was conjugated to OVA and BSA and antibodies were raised both in rabbits and chicken. Endo-1-OVA gave the best antibodies in rabbits whereas Endo-1-BSA was best to raise IgY antibodies. The reason obviously is that OVA is derived from chicken egg and hence may not elicit immunological response. Endo-2 hapten protein conjugates did not give better antibodies and the Endo-diol-HRP conjugate was not useful as tracer.

The titer of the antibody produced in chicken is at an average of 100mg /egg. (Fig. 1.) The eggs are collected for almost 3 months i.e. 90 eggs , 90x 100 mg = 9000mg or 9 g/ hen. Even by conservative measures we take it as 5g/hen. It is almost 70 times more than the yield of the rabbit antibodies. This takes care of the inherent drawbacks of rabbit antibodies such as quantity and reproducibility.

In the chicken the indirect ELISA worked best at 0.04 ug of the antigen and 0.25 ug of the antibody against 1/5k secondary – HRP conjugate dilution. (IC 50 = 10 ppb) (FIG.2). Fig 3 shows the results of the competitive ELISA against 6 HRP – conjugates with an IC 50 value of 100 ppb. These data indicate that the chicken antibody is better than the rabbit antibody.

In the rabbits the competitive assays were most sensitive when DDA-GABA-HRP conjugate was used as tracer while DDT-OH-HRP did not give good response. (FIG.4) (IC 50 = 100 ppb).

By the above paragraphs the inventors respectfully submit that there invention provides pesticidal antibodies which are not taught in the combination of references. These pesticidal antibodies are derived from processes other than disclosed in the combination of references. To start with the inventors wish to state that Examiner should look into the invention as a while. In this regard, the inventors state, that to develop an successful antibody one needs a specific antigen. In case of pathogens the antigens are either the cell wall proteins or inactivated pathogens themselves. What the cited combination of references does not suggest is how or what type of antigen is needed for the pesticide antibodies. Moreover, if the pesticide is used as antigen itself, then it will be toxic for the any bird or animal. The present invention obviates this problem and this has not been suggested by the cited combination of references.

The idea of the present study was not to just make antigens which elicit pesticide antibodies but to make and derive antigens which have dual attributes, i.e first they should be non-toxic to the study system (rabbits or birds) and second should elicit good antibody response. Such kind of results could not be expected from the cited combination of reference. The prior art have even failed to suggest and recommend such studies. This is because in the prior art selection of an antigen was far more obvious and easy, as the functions of cell wall proteins as known to antibody elicitors is well known. But this is not the case for pesticides, which are organic molecules. Instead the inventors had to devise and evolve a new means to creating a pesticide antibody. This has not been taught in US 5,753,228;

US 4,357,272 and US 5,688,682.

The inventors wish to further point out that even the process (i.e. protocol used) in isolating the antibodies is different between the present invention and the prior art. The Examiner would notice that the very reason that the nature and origin of antigens are different makes the process of producing antibodies also different. In other words the antigens of the cited references have cellular origin (i.e. they are proteins), whereas the antigens of the present invention are non-cellular in origin (i.e. they are pesticides).

The Examiner is requested to look into the invention as a whole rather than with a hindsight view. The question is not of just of using the type of animal system but the question lies with the fact how, whether and can the animal system i.e. poultry birds, sustain injection

such as non-cellular antigens for raising the antibodies. The inventors have also used rabbits but they have shown that rabbit antibodies are not as effective as the poultry bird antibodies.

In the present invention the whole process of making pesticide antibodies is interlinked. To develop the novel pesticide antibody, the development of antigens was the key and by no means prior art have designated such fact. There was a lot of experimentation and conscientious planning of protocols to arrive at such results. A lot of analysis went into studying the various intermediates and products during the antigen development. The inventors could not just pick any antigen at random and use it. Many experiments were done before the desired antigen was discovered. The Examiner should keep notice of the fact that the antigens to be injected in the animal system were to be developed from toxic pesticides and a lot care, caution and vigilance were needed. Also, one cannot inject the pesticides directly into the birds, as they are lethal. Therefore, there was a great unfulfilled need to identify and develop specific pesticide antigen to function as effective elicitor for the antibody.

Fig 1 : ESTIMATION OF ANTIBODY TITER

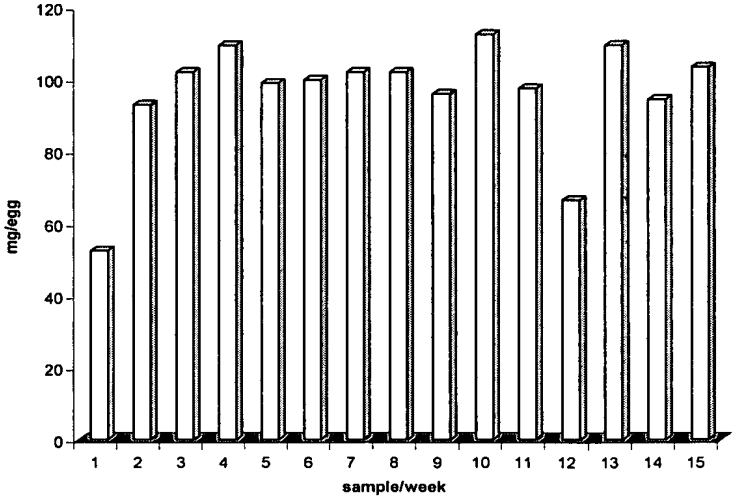


Fig 2: CHECKER BOARD ANALYSIS OF ANTIBODY AND ANTIGEN

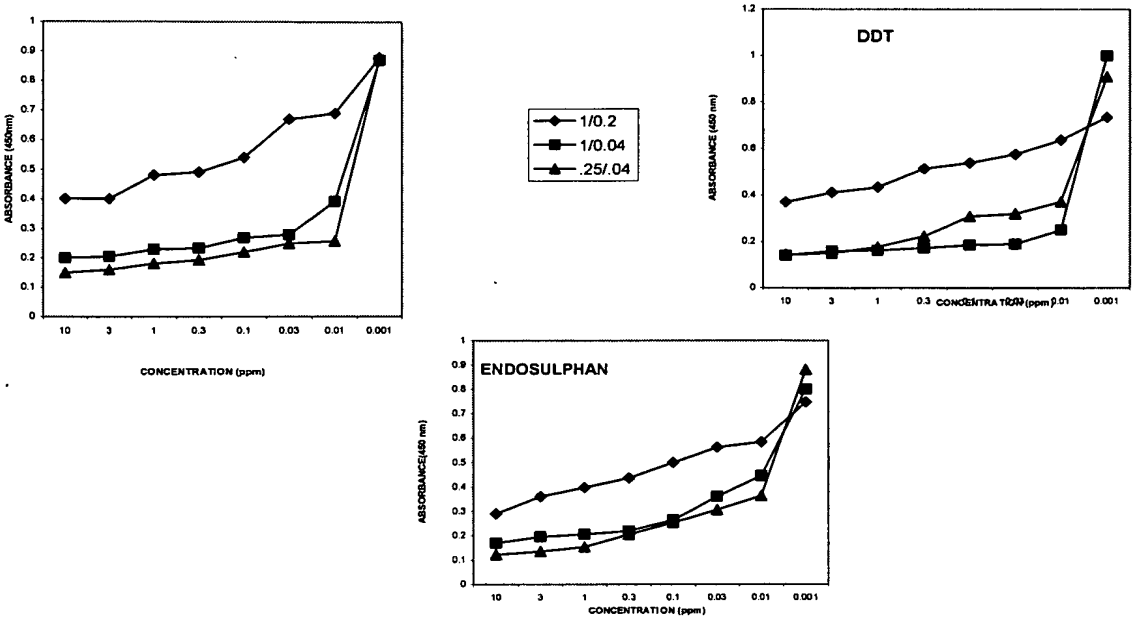


Fig 3: CHECKER BOARD ANALYSIS FOR DDT ASSAY WITH DIFFERENT ENZYME CONJUGATES

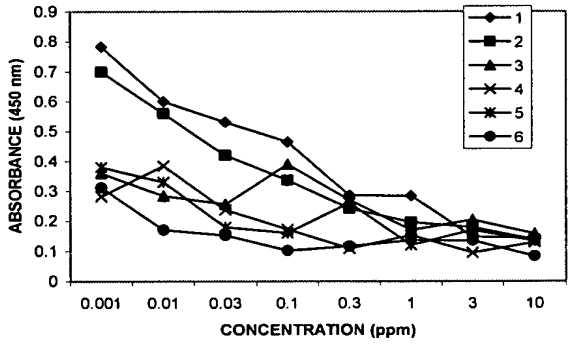
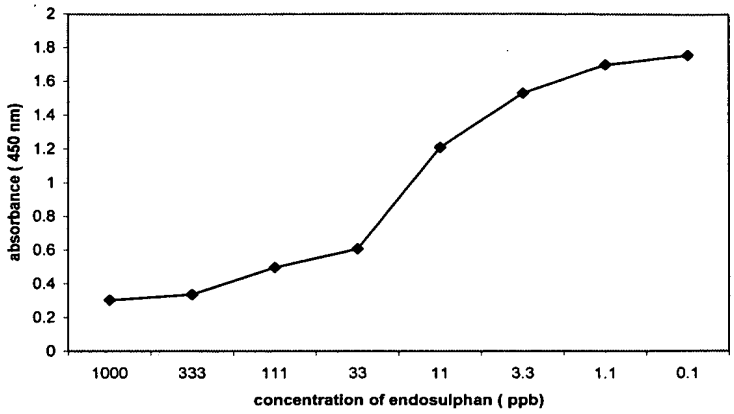


Fig 4 : Standard graph of DDT (rabbit antibodies)



In light of these remarks and the evidence of the 132 Declaration and the arguments previously submitted, applicants urge that one of skill in the art would not have been motivated to combine the references cited by the Examiner. Nor would the skilled artisan have a reasonable expectation of success of the present invention. Finally, the interest in the commercialization of the present invention is evidence of non-obviousness. Therefore, applicants submit the obviousness rejections of record are improper and respectfully request withdrawal of these rejections.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of

papers submitted herewith, Applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date September 21, 2004

FOLEY & LARDNER LLP
Washington Harbour
3000 K Street, N.W., Suite 500
Washington, D.C. 20007-5143
Telephone: (202) 672-5300
Facsimile: (202) 672-5399

Matthew E. Mulkeen
Registration No. 44,250